European Journal of Molecular & Clinical Medicine
ISSN 2515-8260 Volume 07, Issue 11 , 2020

"STUDIES ON ANTIMICROBIAL & ANTIOXIDANT PROPERTIES OF ASHWAGANDHA AROUND NANDED DISTRICTS OF MAHARASHTRA"

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Abstract

In the current study, we evaluate the antibacterial study of Ashwagandha [Withania somnifera L. (Solanaceae; root and leaves), which is an traditional medicinal plant which is used in the treatment of the pathogenic bacteria. to possess strong antibacterial activity against a range of bacteria, as revealed by in vitro Agar Well Diffusion Method, we have used Aqueous as well as alcoholic extracts of the plant (root as well as leaves) were found. The methanolic extract was further sub-fractionated using various solvents and the butanolic sub-fraction was found to possess maximum inhibitory activity against a spectrum of bacteria including Salmonella typhimurium. Moreover, in contrast to the synthetic antibiotic (viz. chloramphenicol), these extracts did not induce lyses on incubation with human erythrocytes, advocating their safety to the living cells. Finally, the antibacterial efficacy of the extracts isolated from plant (both root and leaves) was determined against experimental salmonellosis. Oral administration of the aqueous extracts successfully obliterated salmonella infection which also revealed by increased survival rate as well as less bacterial load in various vital organs of the treated animals.

Key word: Withania somnifera L., Antimicrobial, Antioxidant

Background:

Withania somnifera is an important medicinal plant that has been used in Ayurvedic and indigenous medicine since ancient times. In the view of its varied therapeutic potential, it has also been the subject of considerable modern scientific attention. Attention has been drawn to antibacterial activity of the plant and its metabolites due to the challenge on growing antibacterial resistant pathogens.

Introduction:

Withania somnifera (L.) also known as "Aswagandha" belongs to the family Solanaceae and is widely used in Ayurvedic medicine.[1-2] It is an ingredient in many formulations prescribed for a variety of musculoskeletal conditions (e.g. arthritis, rheumatism), and as a general tonic to increase energy, improve overall health and longevity, and prevent disease in athletes, and elderly. Recently, the antimicrobial activity of W. somnifera was studied by several authors on

control strains (microbial type culture collection).[3-7] However, there is no report of antibacterial activity of leaf extract of W. somnifera on human pathogenic Gram-positive bacterial strains from soft tissue infection. Hence, this study was planned to examine the antimicrobial potential of leaf extract of W. somnifera against Gram-positive cocci (n = 20) from pus samples of patients suffering from soft tissue infection.

Materials and Methods

Plant description

Withania somnifera is an erect, herbaceous, and evergreen tormentor's shrub. The leaf base is cuneate and is densely hairy beneath. The flowers are yellow and berries orange-red.

Plant collection

The leaves of W. somnifera were collected from Different regions of Nanded District.

Preparation of extract

Extraction procedure was followed as Owais et al. with slight modification at the research and development laboratory. Fresh leaves were washed thoroughly 2–3 times with running water followed by sterile distilled water. Washed leaves were air dried under the shade at room temperature and then pulverized by mortar pestle. Shade dried leaf powder was shaken overnight in methanol and then extracted successively in Soxhlet apparatus. Extract was filtered by Whatman number one filter paper and filtered solution was evaporated under reduced pressure with the help of rotary evaporator. The dried leaf extract was dissolved in methanol to final concentration of 1 mg/ml and 2 mg/ml.[8]

Microorganisms

Gram-positive Cocci obtained from clinical samples of pus from patients admitted to Government Medical College, Parbhani were used to find out the antimicrobial potential of leaf extract of W. somnifera. A total of 20 isolates including S. aureus (MRSA and methicillin sensitive staphylococcus aureus [MSSA]), Enterococcus and Streptococcus spp. were examined against methanolic extract of leaf of W. somnifera.

Sensitivity testing

The antibacterial susceptibility test was carried out using the agar diffusion method. Muller Hinton agar (MHA) was used for antibacterial susceptibility testing. For diffusion method, Petri plates were prepared by pouring 20 ml of MHA. Stock bacterial solution was thawed and immediately suspended in peptone water and incubated for 2–3 h at 37°C. After matching the turbidity with 0.5 Mc Farland, the innoculum of each isolate was spread on two MHA plates and was allowed to dry for 10 min. Four wells of 9 mm diameter each were punched in each plate using a sterile borer. Plant extract with concentration of 1 mg/ml and 2 mg/ml with different volumes of 20 µl, 50 µl, and 100 µl were poured in each of the 3 wells of MHA plates containing bacterial inoculum. Fourth (control well) was filled with 50 µl methanol. The plates were kept for 1 h at room temperature to allow the diffusion into the medium and then incubated aerobically at 37°C for 18 h. The inhibition zones formed around the wells were measured in

millimeters. For each concentration, the zones of inhibition produced by different strains of a species were averaged.

Antimicrobial Activity of Withania Somnifera

Minimum inhibitory concentration (MIC) was determined for plant extract showing antimicrobial activity against test pathogens. Broth microdilution method was followed for determination of MIC values. Plant extracts were resuspended in acetone (which has no activity against test pathogens) to make 10 mg/ml final concentration and then two fold serially diluted; and added to broth media of 96-wells of microtiter plates. Thereafter 100 µl inoculum (for bacteria 1×10^8 CFU/ml and 1×10^7 CFU/ml for yeast and fungi) was added to each well. Bacterial and fungal suspensions were used as negative control, while broth containing standard drug was used as positive control. Total activity is the volume at which the test extract can be diluted with the ability to kill the microorganisms. It is calculated by dividing the amount of extract from 1 g plant material by the MIC of the same extract or compound isolated and is expressed in ml/g.

Anti-Oxidant study

Determination of antioxidant activity

ABTS radical scavenging assay

To measure the antioxidant capacity by monitoring inhibition of the $ABTS^{\cdot+}$ radical cation, the method described by Surveswaran et al. was followed. The generation of $ABTS^{\cdot+}$ radical cation was carried out by a chemical reaction between a solution of ABTS (7 mM) and a solution of potassium persulfate (2.45 mM) incubated for 16 h in the dark at room temperature. The resulting $ABTS^{\cdot+}$ solution was diluted with methanol (at a ratio of 1/50) in order to obtain an absorbance of 0.700 ± 0.005 at a wavelength of 734 nm. A volume of 100 µl of plant extract solution prepared at different concentrations (10, 5, 2, 1, 0.66 or 0.4 mg ml^{-1}) was added to 3.9 ml of $ABTS^{\cdot+}$ solution (absorbance equal to 0.700 ± 0.005). After 10 min of reaction, the absorbance was measured by a spectrophotometer (Jenway 6315-United Kingdom) set to 734 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as standard antioxidant at a concentration range of 0–500 µM. The results were expressed in µM Trolox equivalent per gram of extract (µM TE g^{-1}).[9]

DPPH radical scavenging assay

The antiradical activity of plant extracts was determined by the DPPH radical scavenging assay according to the method adopted by Orphanides et al. A volume of 3.9 ml of $DPPH^{\cdot}$ solution (0.3 mM) was mixed with 100 µl of plant extract prepared at different concentrations (10, 5, 2, 1, 0.66 or 0.4 mg ml^{-1}) and incubated for 30 min at room temperature. The absorbance were then measured by a spectrophotometer (Jenway 6315-United Kingdom) set to 517 nm. Trolox was used as a standard antioxidant at a concentration range of 0–500 µM. The results were expressed in µM TE g^{-1}.[10]

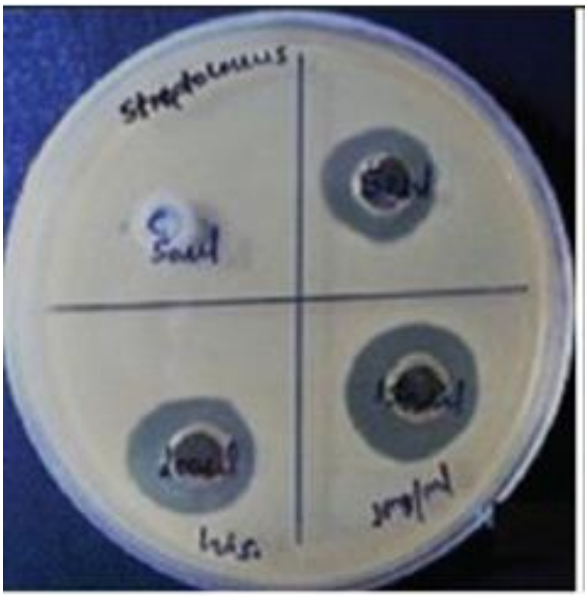
Ferric reducing ability (FRAP)

The ability of the various extracts to reduce ferric iron (Fe^{+3}) to ferrous iron (Fe^{+2}) was determined according to the protocol described by Orphanides et al. Dilutions of plant extracts

and Trolox were prepared at the same concentrations as used in the antioxidant assays above. A volume of 100 µl of extract was mixed with 3.9 ml of a freshly prepared FRAP solution [0.3 M acetate buffer, pH = 3.6, 10 mM TPTZ (2,4,6-tri-(2-pyridyl)-s-triazine) and 20 mM $FeCl_3 \cdot 10H_2O$ at a ratio of 10:1:1 (v/v/v)]. The mixture was then incubated at 37 °C for 4 min, and the absorbance was measured at a 593 nm. Trolox was used as a standard antioxidant at a concentration range of 0–800 µM. The results were expressed in µM TE g^{-1}. [10]

Results and Discussion:

Methanolic root extracts of W. somnifera revealed 4 mm inhibitory zone against Escherichia coli and 10 mm inhibitory zone at 10 µg/ml against Enterococcus, which is very less than that observed in our study. This difference may be due to a different part of the plant extract being used. Owais et al., observed 22 mm zone of inhibition against S. aureus at a concentration of 20 mg/ml of W. somnifera leaf extract. In another study, 15 mm zone of inhibition at a concentration of 100 µg/ml against S. aureus and E. coli by W. somnifera leaf extract was reported. In present study good inhibitory zone against Enterococcus spp., and S. aureus at the concentration of 2 mg/ml was observed. Difference in the zone of inhibition might be attributed to the difference in bacterial strains used, methodology employed, and different geographical area from where the plant was obtained (fig.1).



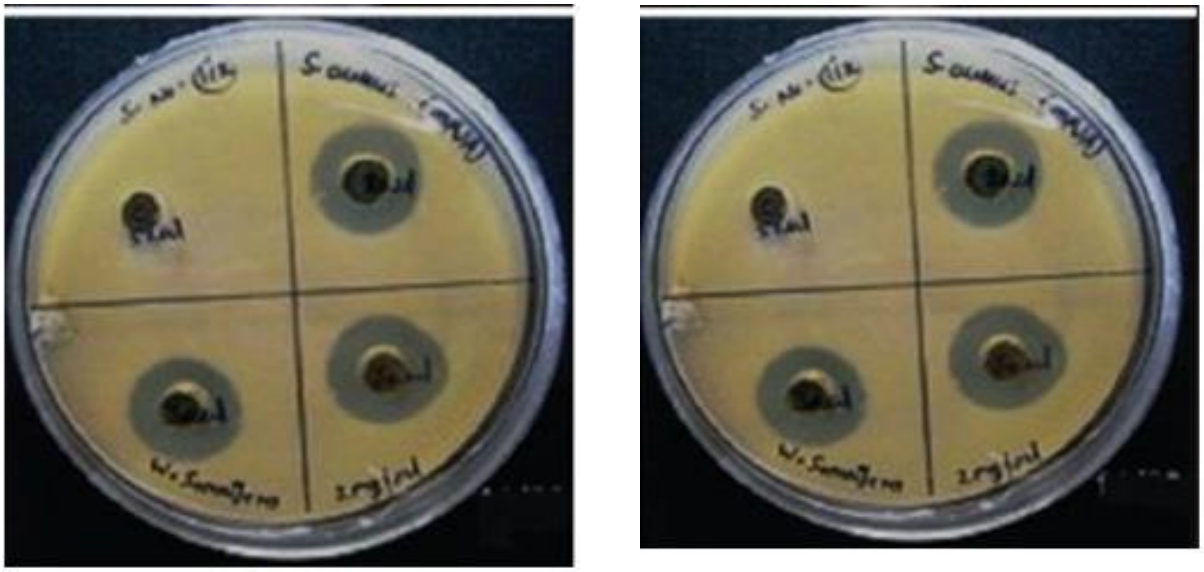


Fig. 1: Zone of inhibition (in mm) by Withania somnifera leaf extract in concentration of 1 mg/ml by using different volumes of 20 µl, 50 µl, and 100 µl and 2 mg/ml by using different volumes of 20 µl, 50 µl, and 100 µl on pathogenic isolates of -Staphylococcus aurous and Streptococcus spp. C-negative control (containing 100 µl methanol)

Activity index for each extract was calculated (**Table 1**) as, Activity index=IZ produced by extract/IZ produced by standard; where IZ is inhibition zone.

Table 1: Activity index for each extract

Plant part	**Extract**	**Test microorganism**									
		P. aeruginosa		**E. coli**		**P. mirabilis**		**S. aureus**		**C. albicans**	
		IZ (mm)	**AI**	**IZ (mm)**	**AI**	**IZ (mm)**	**AI**	**IZ (mm)**	**AI**	**IZ (mm)**	**AI**
Root	E_1	-	-	-	-	-	-	12.5	0.595 ±0.014	7.83	0.559 ±0.031
	E_2	10	0.500	25.5	0.980±0.	28.5	1.140	21.5	1.023	30	2.142

Plant part	Extract	Test microorganism									
		P. aeruginosa		E. coli		P. mirabilis		S. aureus		C. albicans	
		IZ (mm)	AI	IZ (mm)	AI	IZ (mm)	AI	IZ (mm)	AI	IZ (mm)	AI
			±0.050		011		±0.012		±0.014		±0.041
Stem	E_1	-	-	-	-	11.66	0.467 ±0.012	11	0.523 ±0.027	10.16	0.726 ±0.031
	E_2	-	-	-	-	-	-	8.5	0.404 ±0.024	8.5	0.607 ±0.020
Leaf	E_1	-	-	16.5	0.634±0.033	10	0.400 ±0.012	10.5	0.499 ±0.024	19.5	1.392 ±0.020
	E_2	-	-	-	-	11.5	0.460 ±0.012	15.33	0.729 ±0.124	8	0.571 ±0.021
Fruit	E_1	-	-	-	-	16.5	0.660 ±0.035	-	-	10	0.714 ±0.021
	E_2	-	-	-	-	-	-	13	0.618 ±0.090	12.5	0.892 ±0.02

Antimicrobial activity (assessed in terms of inhibition zone and activity index) of the plant extracts, tested against selected microorganisms were recorded. In the present study total eight extracts of different parts of plant were tested for their bioactivity.

MIC and MBC/MFC values (**Table 2**) were evaluated for plant extracts which had shown activity in diffusion assay. The range of MIC and MBC/MFC of extracts recorded was 0.039-0.625 mg/ml and 0.039- 1.25 mg/ml, respectively. In the present investigation lowest MIC value 0.039 mg/ml was recorded against E. coli, P. mirabilis, S. aureus and C. albicans whereas, against P. aeruginosa lowest MIC observed was 0.156 mg/ml, indicating significant antimicrobial potential of test extract. MIC and MBC/MFC values were found to be same for extracts of plant.

Table 2: Mic and Mbc/Mfc Values of W. Somnifera against Test Pathogens

Plant	**Extract**	**Test microorganism**									
part		**P. aeruginosa**		**E. coli**		**P. mirabilis**		**S. aureus**		**C. albicans**	
		MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MFC**
Root	E_1	-	-	-	-	-	-	0.156	0.312	0.312	1.25
	E_2	0.156	0.312	0.039	0.039	0.039	0.039	0.039	0.078	0.039	0.039
Stem	E_1	-	-	-	-	0.078	0.156	0.156	0.312	0.078	0.156
	E_2	-	-	-	-	-	-	0.625	0.125	0.156	0.312
Leaf	E_1	-	-	0.078	0.078	0.312	0.312	0.156	0.312	0.078	0.078

Plant part	**Extract**	**Test microorganism**									
		P. aeruginosa		**E. coli**		**P. mirabilis**		**S. aureus**		**C. albicans**	
		MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MFC**
	E_2	-	-	-	-	0.156	0.312	0.078	0.156	0.156	0.312
Fruit	E_1	-	-	-	-	0.078	0.078	-	-	0.078	0.156
	E_2	-	-	-	-	-	-	0.078	0.156	0.039	0.039

E1 - Free flavonoids; E2 - Bound flavonoids; MIC - Minimum inhibitory concentration (mg/ml); MBC/MFC - Minimum bactericidal/fungicidal concentration (mg/ml)

Quantity of extract obtained per gram from plant parts and total activity (TA) was calculated and recorded (**Table 3**). Total activity indicates the volume at which extract can be diluted without loosing ability to kill microorganism. Most of the extracts showed high values of TA against E. coli, P. mirabilis, S. aereus and C. albicans. Maximum TA values calculated were 38.46, 153.84, 198.71, 153.84 and 198.71 ml against P. aeruginosa, E. coli, P. mirabilis, S. aureus and C. albicans, respectively.

Table 3: Quantity of extract obtained per gram from plant parts and total activity (TA)

Plant	Extract	Amount of extract mg/gdried plant part	Total activity (ml/g)				
Part			P. aeruginosa	E. coli	P. mirabilis	S. aureus	C. albicans
Root	E_1	7	-	-	-	44.87	22.43

Plant	Extract	Amount of extract mg/gdried plant part	Total activity (ml/g)				
Part			P. aeruginosa	E. coli	P. mirabilis	S. aureus	C. albicans
	E_2	6	38.46	153.84	153.84	153.84	153.84
Stem	E_1	5.5	-	-	70.51	35.25	70.51
	E_2	3	-	-	-	4.80	19.23
Leaf	E_1	10.5	-	134.61	33.65	67.30	134.61
	E_2	3.5	-	-	22.43	44.87	22.43
Fruit	E_1	15.5	-	-	198.71	-	198.71
	E_2	6.5	-	-	-	83.33	166.66

E1 - Free flavonoids; E2 - Bound flavonoids; total activity = Extract per gram dried plant part/MIC

Quantity and Total Activity of Free and Bound Flavonoids of W. Somnifera

There is a continuous and urgent need to discover new antimicrobial compounds as there is an alarming increase in the incidence of new and re-emerging infectious diseases. Medicinal plants could be a good alternative source for costly antibiotics (against which microbes are developing resistance rapidly), as most of the medicinal plants are safe with little or no side effects, cost effective and have ability to affect a wide range of antibiotic resistant microorganisms.

Present study is an effort towards this direction. In the present study IZ, AI, MIC, MBC/MFC and TA have been evaluated for each extract. For most of the extracts MIC values recorded were very low, indicating strong bio efficacy of the plant. Most of the extracts of plant were found to be potent inhibitor of tested microorganisms except P. aeruginosa, against which only one extract of the plant showed activity. Excellent activity was shown by bound flavonoids of roots having low MIC and MBC/MFC values.

Extracts with higher MBC/MFC values than MIC values against microorganisms tested, indicate the bacteriostatic/fungistatic effects of the extracts. Bound flavonoids of roots were found to be

bactericidal against E. coli and P. mirabilis and fungicidal against C. albicans. Bound flavonoids of fruit were found fungicidal against C. albicans. Free flavonoids of leaf were found bactericidal against E. coli and P. mirabilis and fungicidal against C. albicans whereas, free flavonoids of fruit were found bactericidal against P. mirabilis.

Extracts under study not only inhibit the bacterial/ fungal growth but the IZ developed, was more or less permanent when compared with the IZ developed by the standard drug used, as after sometime bacterial/fungal colonies could be easily seen in IZ developed by standard drugs. In the light of the fact that microorganism are becoming resistant against the drugs in use, present investigation is of great significance, as far as the future drugs are concerned and advocates uses of selected plant by the pharmaceutical industries for preparing plant based antimicrobials drugs.

The antioxidant activity of methanolic and aqueous extracts of the plants was determined in vitro by three techniques, namely, the ABTS, DPPH and FRAP assays. According to the results shown in Figs. 7, 8 and 9, the plant extracts exhibited remarkable antioxidant activity expressed in µM TE g^{-1}. The methanolic and aqueous extracts of W. somnifera showed the highest antioxidant activity in the FRAP assay; their reducing powers were 6080.67 µM TE g^{-1} and 5315.11 µM TE g^{-1}, respectively.

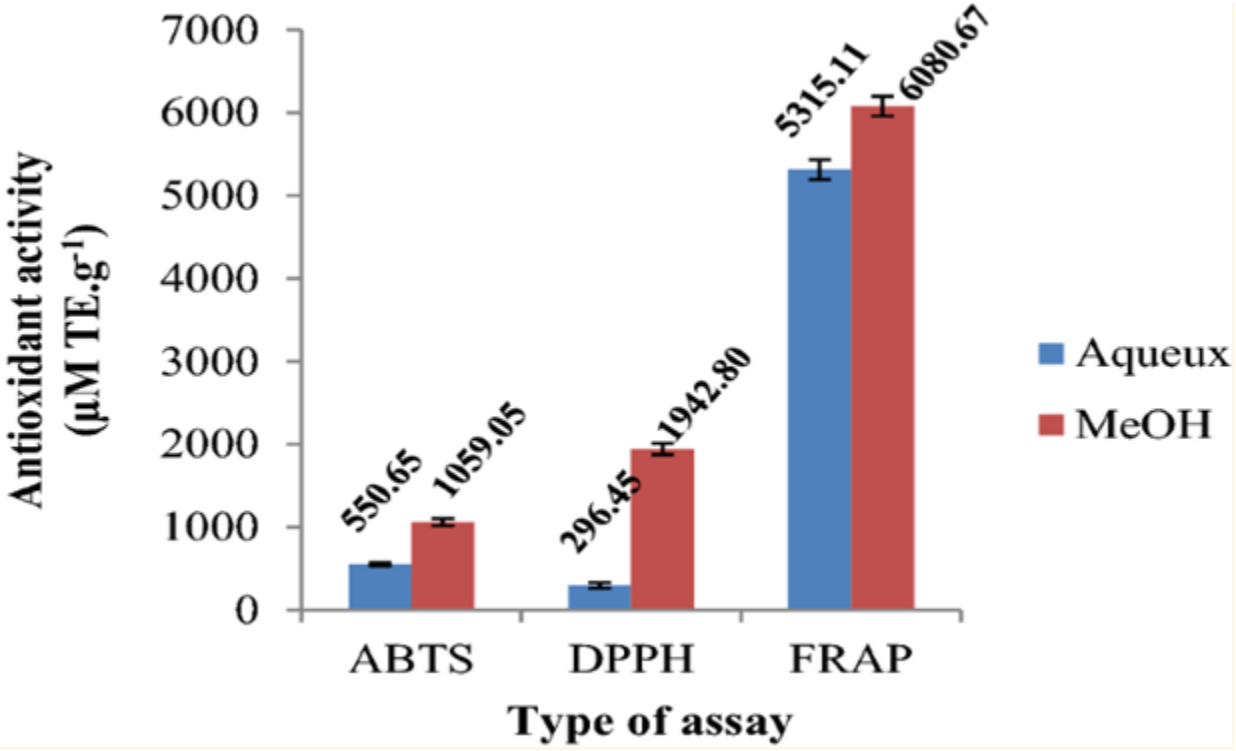


Fig. 2: Antioxidant activity of W. somnifera methanolic and aqueous extracts

For all extracts, the lowest IC_{50} were observed in the methanolic extracts of W. somnifera in the FRAP assay, and IC_{50} values was 0.91 ± 0.92 mg ml^{-1} determined by the FRAP assay. For aqueous extracts, W. somnifera has the highest antioxidant potential with a lower IC_{50} (2.42 ± 0.11). However, the values of antioxidant activities evaluated by ABTS, DPPH and

FRAP assays of all extracts were lower than the standard antioxidant used in this study (Trolox) (Table 4).

Table 4: IC50 of W. somnifera aqueous and methanolic extracts obtained by FRAP, ABTS and DPPH assay

	ABTS		FRAP		DPPH	
	W. somnifera	Trolox	W. somnifera	Trolox	W. somnifera	Trolox
IC_{50} (mg ml^{-1}) of aqueous extract	6.74 ± 0.84	0.98 ± 0.1	2.42 ± 0.11	0.35 ± 0.04	7.58 ± 0.22	0.25 ± 0.03
IC_{50} (mg ml^{-1}) of methanolic extract	5.13 ± 0.63		0.91 ± 0.92		3.27 ± 0.64	

Variance analyses indicated that there are highly significant differences ($P < 0.01$) among the results of three antioxidant activity assays for the same species. Differences were also recorded among the three studied species ($P < 0.05$). The observed differences in the antioxidant activity among the different plant species is probably due to differences in their chemical composition. The differences among the assays seem to be due to the use of a different reaction mechanism by each assay.

Conclusion: The species of Withania Somnifera Leaf extract is resistant pathogens by alternative systems of medicine. Clinical trials with Withania somnifera for its activity against bacterial infections should be conducted.

Our results clearly indicate that W. somnifera, particularly the leaves, has remarkable antioxidant properties. Additionally, the leaves possess significant antibacterial properties against Gram-negative organisms, in particular, S. aurous. It will be beneficial to investigate the active compounds present in W. somnifera so that its leaves can be used to increase the armamentarium of antimicrobial agents and so that other possible therapeutic uses of the plant can be explored.

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